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# Mitochondrial DNA mutations cause resistance to opening of the permeability transition pore

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**Abstract**

The age-related accumulation of mitochondrial DNA mutations has the potential to impair organ function and contribute to disease. In support of this hypothesis, accelerated mitochondrial mutagenesis is pathogenic in the mouse heart, and there is an increase in myocyte apoptosis. The current study sought to identify functional alterations in cell death signaling via mitochondria. Of particular interest is the mitochondrial permeability transition pore, opening of which can initiate cell death, while pore inhibition is protective. Here, we show that mitochondria from transgenic mice that develop mitochondrial DNA mutations have a marked inhibition of calcium-induced pore opening. Temporally, inhibited pore opening coincides with disease. Pore inhibition also correlates with an increase in Bcl-2 protein integrated into the mitochondrial membrane. We hypothesized that pore inhibition was mediated by mitochondrial Bcl-2. To test this hypothesis, we treated isolated mitochondria with Bcl-2 antagonistic peptides (derived from the BH3 domain of Bax or Bid). These peptides released the inhibition to pore opening. The data are consistent with a Bcl-2-mediated inhibition of pore opening. Thus, mitochondrial DNA mutations induce an adaptive–protective response in the heart that inhibits opening of the mitochondrial permeability pore.


*Keywords:* Apoptosis; mtDNA; Bcl-2; Mitochondria; Calcium; Permeability transition pore

## 1. Introduction

Mitochondria contain their own genome which accumulates mutations with age [1]. Further, levels of mitochondrial DNA mutations are elevated in disease states, including heart disease [2,3]. This correlative information suggests that mitochondrial DNA mutations may contribute to senescence and disease. In support of this hypothesis, accelerated mutagenesis of the mitochondrial genome in the heart leads to heart disease [4].

Interestingly, mitochondrial respiratory function remains intact, as would be expected given the low level of mutations [4]. Further, oxidative stress does not increase, contrary to the hypothesized vicious cycle [5]. One proposed mechanism by which mitochondrial DNA mutations may contribute to disease is through apoptosis. Mitochondria regulate the intrinsic pathway of apoptosis through the release of apoptogenic factors, for instance cytochrome *c* [6]. Cytochrome *c* can be released through opening of the mitochondrial permeability transition pore [7]. The pore is highly regulated, [8,9] pore opening can trigger cell death, [10] and inhibition of pore opening can prevent cell death [11].

To study the effects of accumulating mitochondrial DNA mutations, we previously generated a transgenic mouse model [4]. Transgenic mice expressed a proofreading-deficient mitochondrial DNA polymerase in the heart, and accumulated mitochondrial DNA mutations at an accelerated rate. These mice developed heart disease and a prominent aspect of the pathology was increased cell death, including increased release of mitochondrial cytochrome *c* [12].

Further, we observed increased Bcl-2 levels in hearts of transgenic mice [5]. The Bcl-2 family proteins all share at least one Bcl-2 homology domain (BH-domain). Antiapoptotic members have BH domains 1–4, while proapoptotic members contain BH 1–3. The "BH3-only" family members are unrelated except at the BH3 domain (for review, see Ref. [13]). Bcl-$_{XL}$ and Bcl-2 inhibit pore opening induced by calcium, [14,15] and this function is

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countered by proapoptotic Bcl-2 family members [16,17]. Thus, there is a balance of pro- and antiapoptotic Bcl-2 family members at the mitochondrial membrane regulating pore opening and cytochrome *c* release.

The confluence of apoptosis and Bcl-2 upregulation in mice with mitochondrial lesions led us to study the function of the mitochondrial permeability transition pore. Specifically, we examined calcium-induced pore opening, mitochondrial calcium uptake, and Bcl-2-mediated pore inhibition.

## 2. Materials and methods

### 2.1. Animals

The transgenic mice used in this study express a proofreading-deficient mitochondrial DNA polymerase under direction of the heart-specific a-myosin heavy chain promoter and have been described [4]. They were maintained as two independent lines, hemizygous animals were studied and always compared to wildtype littermate controls. No differences in results were noted between the two transgenic lines. All procedures were approved by the Institutional Animal Care and Use Committee.

### 2.2. Mitochondrial isolation

Cardiac mitochondria were isolated by differential centrifugation [18] in isolation buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM 4-morpholenepropanesulfonic acid (MOPS), 0.5% fatty acid-free BSA, pH 7.2 with potassium hydroxide). The pellet was then washed and mitochondrial ADP was depleted by treatment for 5 min at 30 °C with 2 mM pyrophosphate in isolation buffer [19]. Mitochondria were washed twice with resuspension buffer (250 mM sucrose, 0.1 mM EGTA–tris, 10 mM Tris–HCl, pH 7.4), and then resuspended at approximately 20 mg of mitochondrial protein/mL (proteins by BCA). Hearts from multiple animals (usually 4–6) were processed together to ensure adequate mitochondria for each experiment. In the figure legends, "n" refers to the number of independent mitochondrial preparations tested.

### 2.3. Respiration

Mitochondria (50 μg) were added to 1 mL respiration buffer (250 mM sucrose, 10 mM phosphoric acid–tris, 5 μM EGTA–tris, 10 mM MOPS–tris, pH 7.3) that contained 250 nM JC-1 (Molecular Probes, Eugene, OR). Complex I activity was stimulated by the addition of 5 mM pyruvate, 5 mM malate, and 5 mM glutamate (pH 7.5), and complex II was stimulated by 5 mM succinate (pH 8). Rotenone was used to inhibit complex I at 2 μM, potassium cyanide was used to inhibit complex IV at 2 mM, and oligomycin was used to inhibit complex V at 0.25 μg/mL. Fluorescence of JC-1 was monitored every minute using an excitation wavelength ($\lambda_{ex}$) of 490 nm and emission wavelength ($\lambda_{em}$) of 530 nm, followed immediately by a reading at $\lambda_{ex}$ 490 nm, $\lambda_{em}$ 590 nm. The delay between the readings was approximately 4 s. The readings for each time point were converted to fluorescence ratio by dividing the signal at $\lambda_{em}$ 590 nm by the signal at $\lambda_{em}$ 530 nm.

### 2.4. Pore opening, cuvette

Mitochondria (50 μg/mL final) were added to respiration buffer that contained 250 nM JC-1, 2 μM rotenone, 0.25 μg/mL oligomycin, and 5 mM succinate. JC-1 fluorescence readings were taken every minute as above, and the fluorescent ratio was converted into normalized ratio using time zero as 0% and the time point immediately before the addition of calcium as 100%. The ratio at time zero was similar to the value obtained after respiration inhibition by cyanide. The ratio just before calcium addition varied from run-to-run and experiment-to-experiment, and is indicated in the figure legends. The addition of calcium is indicated in the figures. Calcium stocks were prepared fresh daily.

Calcium is known to induce a transient depolarization in mitochondria due to its electrophoretic accumulation. This transient depolarization was seen in multiple traces (not shown) as a reversible decrease in the normalized ratio of JC1 lasting for 1 to 4 min. This decrease was not seen in all experiments, and is thus not apparent in Figs. 1 and 2.

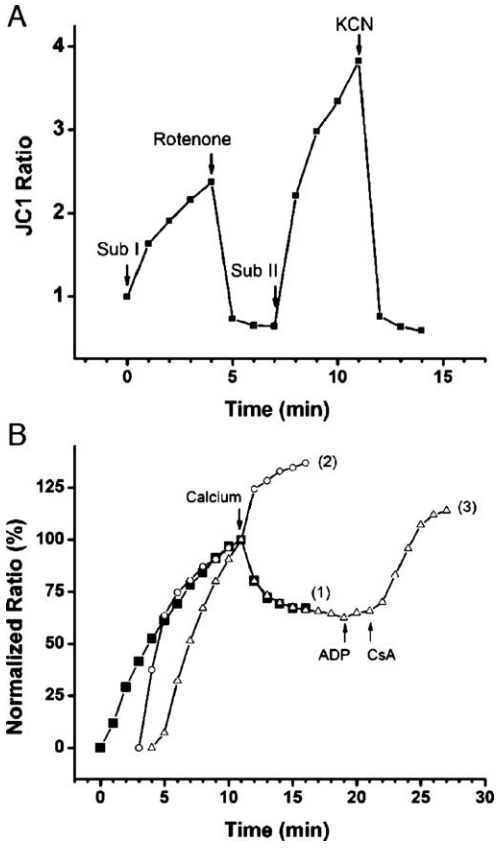



Fig. 1. Fluorescence measurements of mitochondrial respiratory function. (Panel A) Mitochondria (50 μg/mL) were suspended in respiration buffer, and respiratory function was assayed using the lipophilic cation JC-1. As the transmembrane potential increases upon charging with substrates of complex I (Sub I) or II (Sub II), so does the ratio of emitted fluorescence (590 nm/530 nm). The complex I inhibitor rotenone and the complex IV inhibitor cyanide (KCN) arrest respiration, and the transmembrane potential quickly dissipates. (Panel B) Mitochondria were charged with succinate and pore opening was activated by calcium (25 μM; curve 1; solid square). Pore opening and loss of potential can be prevented (curve 2; open circle) or reversed (curve 3; open triangle) by the pore inhibitor cyclosporin A in the presence of ADP. The actual JC-1 ratio was converted as in Materials and methods to facilitate comparison. In Panel B, the average ratio at calcium addition for the 3 runs was 5.42 ± 1.79.

### 2.5. Pore opening, 96-well plate

For some replicates, assays were performed in microtiter plates. Conditions were as above, except that the final volume was 200 μL. Mitochondrial suspensions were prepared and then aliquoted into a black 96-well plate and fluorescence readings taken every minute. At the start of the experiment and after every addition, the plate was shaken (orbital) for 5 s. Because the reactions were prepared in bulk, aliquoted, and shaken before the first time point, the fluorescence reading at "time zero" was slightly delayed compared to runs using cuvettes. Another minor difference was that readings were taken at $\lambda_{ex}$ 485 nm, $\lambda_{em}$ 535 and 595 nm, and the delay between reading the $\lambda_{em}$ at 535 nm and 595 nm was about 15 s. Conditions were otherwise the same.

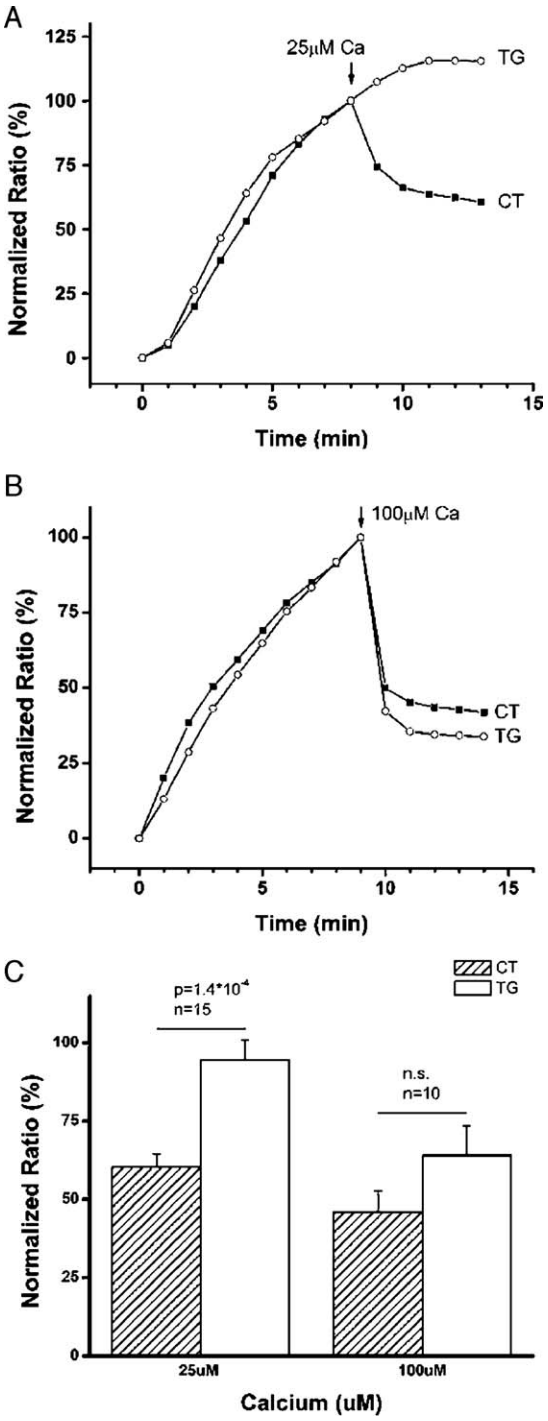
### 2.6. Western blots

The protein levels of Bcl-2 were determined by Western blot of 50 μg mitochondrial protein (antibody from Santa Cruz, CA). Equal loading was

confirmed by probing the blot for the mitochondrial VDAC protein (antibody from Calbiochem, La Jolla, CA). Primary antibody was detected by horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA), followed by detection by ECL-plus (Amersham, Piscataway, NJ).

### 2.7. BH3 peptides

Peptides were obtained from Research Genetics (now Invitrogen, Carlsbad, CA) corresponding to the BH3 domain of human Bax and human Bid proteins, see Fig. 6 for sequences. These peptides were resuspended in degassed $dH_2O$ and stored at −20 °C. Peptide was added to the respiration buffer before mitochondria, and the rest of the assay was identical to that described above.




### 2.8. Calcium uptake

Calcium uptake was measured in respiration buffer with 5 mM succinate, as described [20]. Calcium concentration was obtained using the binding constant and maximal fluorescence ($F_{max}$) values obtained by the double-reciprocal plot of 1/fluorescence versus 1/[calcium] in assay mix without mitochondria. The binding constant of Calcium Green 5N was determined to be 4.8 μM which is close to the expected value (14 μM; Molecular Probes, Eugene, OR).

### 2.9. Statistics

Comparisons are expressed as mean±S.E.M., and significant differences determined using two-tailed Student's *t* test except in experiments using BH3 peptides where multiple comparisons were possible. Then, ANOVA was used with Bonferroni post hoc correction, significance defined at $P<0.05$.

## 3. Results

### 3.1. Transmembrane potential

In mice with mitochondrial DNA mutations we see cytochrome *c* release and cell death [12] and we hypothesized that altered function of the mitochondrial permeability transition pore may be the cause. Thus, we assessed the function of the pore. First, we validated our method of measuring transmembrane potential. Fig. 1A demonstrates mitochondrial respiratory function using the lipophilic cation JC-1 as an indicator of transmembrane charge. When mitochondrial respiration was initiated by the addition of NADH-generating substrates (complex I substrates; Sub I), the emitted fluorescence from JC-1 changed, such that the 590 nm emission increased and the 530 nm emission decreased, graphed as the ratio of 590 to 530 nm emission. These changes are due to the membrane potential-driven accumulation of the dye in the mitochondrial matrix which forms J-aggregates (fluorescence emission at 590 nm) and the resultant depletion of the monomeric form (fluorescence emission at 530 nm) [21]. This fluorescence change was reversed upon inhibition of complex I with rotenone. Regeneration of the potential using the complex II substrate succinate (Sub II) again resulted in J-aggregate formation, which was reversed by inhibiting electron transport downstream of complex II by potassium cyanide inhibition of complex IV. Thus, JC-1 tracks the mitochondrial transmembrane potential in this system.

Fig. 2. Mitochondrial pore function from mice with mitochondrial DNA mutations. (Panel A) At low calcium concentrations (25 μM; 500 nmol/mg mitochondrial protein), control mitochondria (CT) undergo pore opening while mitochondria from transgenic mice (TG) are protected. Concentration dependence studies (not shown) demonstrated that the optimal difference was at 25 μM calcium, and decreased with higher calcium. The JC-1 ratio at calcium addition was 2.97 for control and 2.73 for transgenic. (Panel B) At 100 μM calcium (2000 nmol/mg mitochondrial protein), both control (CT) and transgenic (TG) mitochondria undergo similar, and maximal pore opening. The JC-1 ratio at calcium addition was 2.57 for control and 3.0 for transgenic. Note that in panels A and B, curves reached a plateau by 5 min after the calcium pulse was delivered. The relative charge at this time was compared between multiple independent experiments and the results are shown in panel C. The protection in TG mitochondria is highly significant at 25 μM calcium and is overcome with higher (100 μM) challenge, the average JC-1 ratio at calcium addition was 4.43±0.24.

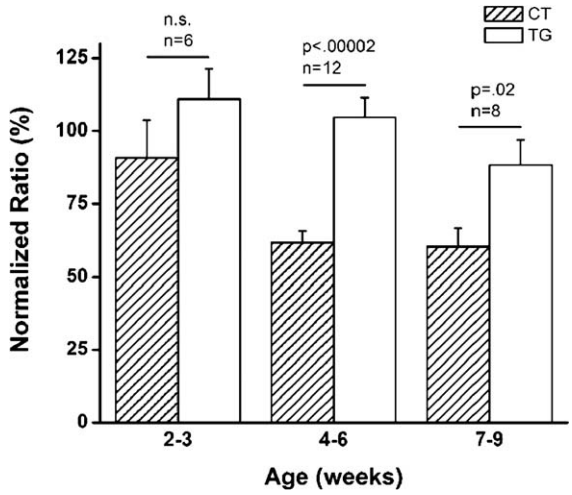



Fig. 3. Mitochondrial pore function with age. Mitochondria (50 μg) were challenged with 25 μM calcium as in Fig. 2. The plateau value 5 min after the calcium pulse was compared over several mitochondrial preparations isolated from mice of different ages. Mice aged 2–3 weeks ($n=6$) are considered "pre-disease," 4–6 weeks ($n=12$) is the onset of disease, measured by gross dilation, and mice aged 7–9 weeks ($n=8$) have had disease for a few weeks. Note that before onset of disease, there is no difference in pore function of CT and TG mitochondria. At about the same time as disease onset, the difference is highly significant, and remains significant thereafter. The average JC-1 ratio at calcium addition was 4.61±0.21.

### 3.2. Mitochondrial permeability transition pore

Opening of the mitochondrial permeability transition pore allows the influx of protons into mitochondria through opening of a large, non-specific pore [22]. Pore opening thus collapses the mitochondrial proton gradient, and can be detected with JC-1. JC-1 was used because it is sensitive and small amounts of mitochondria (such as that isolated from young mouse hearts) can be used. We studied the function of the pore in isolated cardiac mitochondria (Fig. 1B). Mitochondria were charged with succinate in the presence of rotenone and oligomycin. Oligomycin was added to prevent the formation of ATP from ADP so the latter could be used to inhibit the pore. Rotenone, a complex I inhibitor, was included to ensure that charging was via complex II. Pore opening was then triggered by calcium addition (25 μM final; arrow) and measured as a drop in potential caused by the influx of protons through the pore (curve 1). The drop was attributed to opening of the permeability transition pore because cyclosporin A (CsA) in the presence of ADP was able to prevent the calcium-induced loss of membrane potential (curve 2). Further, the drop was not due to inhibition of electron transport, as pore closure using CsA plus ADP (curve 3) or calcium chelation by EGTA (not shown) resulted in prompt restoration of the transmembrane potential.

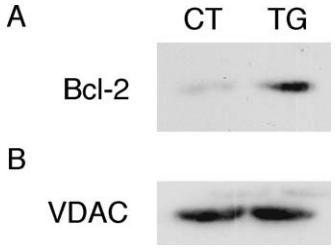



Fig. 5. Bcl-2 at the mitochondrial membrane. Panel A. Western blotting of mitochondria from 6-week-old mice showed that the antiapoptotic Bcl-2 protein was increased on the mitochondrial membrane (blot representative of at least three independent preparations). Panel B. Western blot for VDAC was used to confirm equal protein loading.

Next, we assessed the function of the pore in mitochondria isolated from mice with mitochondrial DNA mutations or from littermate controls. It was expected that mitochondria from mice with mitochondrial DNA mutations would be more sensitive to pore opening, consistent with increased levels of apoptosis and cytochrome *c* release. Interestingly, mitochondria from 5-week-old transgenic animals actually resisted calcium-induced pore opening (Fig. 2A). This resistance was much stronger at low calcium concentrations (25 μM); at 100 μM calcium resistance

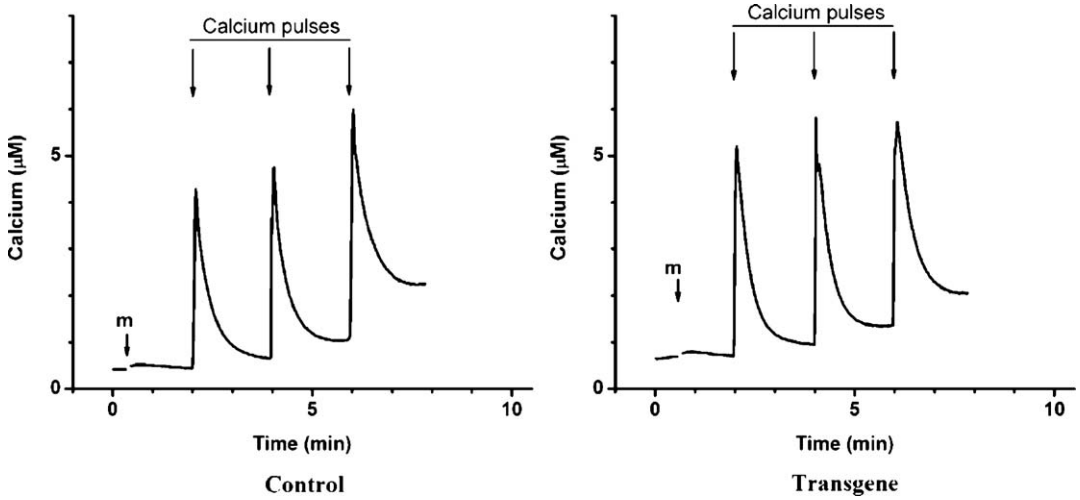



Fig. 4. Mitochondrial calcium uptake. Mitochondria ("m"; 500 μg protein) isolated from 6-week-old mice were added to 1 mL respiration buffer stirring at 30 °C, containing succinate and 1 μM Calcium Green 5N. Fluorescence of this calcium indicator was followed in real time and converted to calcium concentration. Calcium pulses (5 μM) were added every 2 min, and calcium uptake is indicated by decreasing fluorescence. Control experiments (not shown) confirmed that calcium was taken up into the matrix via the calcium uniporter (inhibitable with ruthenium red, an inhibitor of the mitochondrial calcium uniporter), and that uptake was dependent upon a transmembrane potential (inhibitable with cyanide).

was overcome (Fig. 2B). When the relative extent of pore opening (at 5 min after calcium addition) was compared over multiple preparations of mitochondria (Fig. 2C), the resistance at low calcium was highly significant, while at higher calcium, there was no significant difference. We note that pore opening induced by the higher concentration of calcium was still sensitive to cyclosporin A (not shown), thus pore function is not abolished in mice with mitochondrial DNA mutations, only inhibited.

We first detected mitochondrial DNA mutations in mice at 1 week of age [4,23] followed by cytochrome *c* release and cell death at three to 4 weeks-of-age [12]. Bcl-2 upregulation similarly occurred at about 4 weeks-of-age [5]. Finally, dilation of the hearts coincided with cell death, cytochrome *c* release, and Bcl-2 upregulation [12]. Fig. 3 shows that pore dysfunction is present by 4 weeks-of-age, similar to the onset of dilation, Bcl-2 upregulation, and cell death.

A

| | | | |
|---|---|---|---|
| hBid | 82 | IIRNI ARHLA QVGDS MDRSI | 101 |
| G94E | | IIRNI ARHLA QV**E**DS MDRSI | |
| hBax | 55 | STKKL SECLK RIGDE LDSNM | 74 |
| ΔGD Bax | | STKKL SECLK RIELD SNM | |

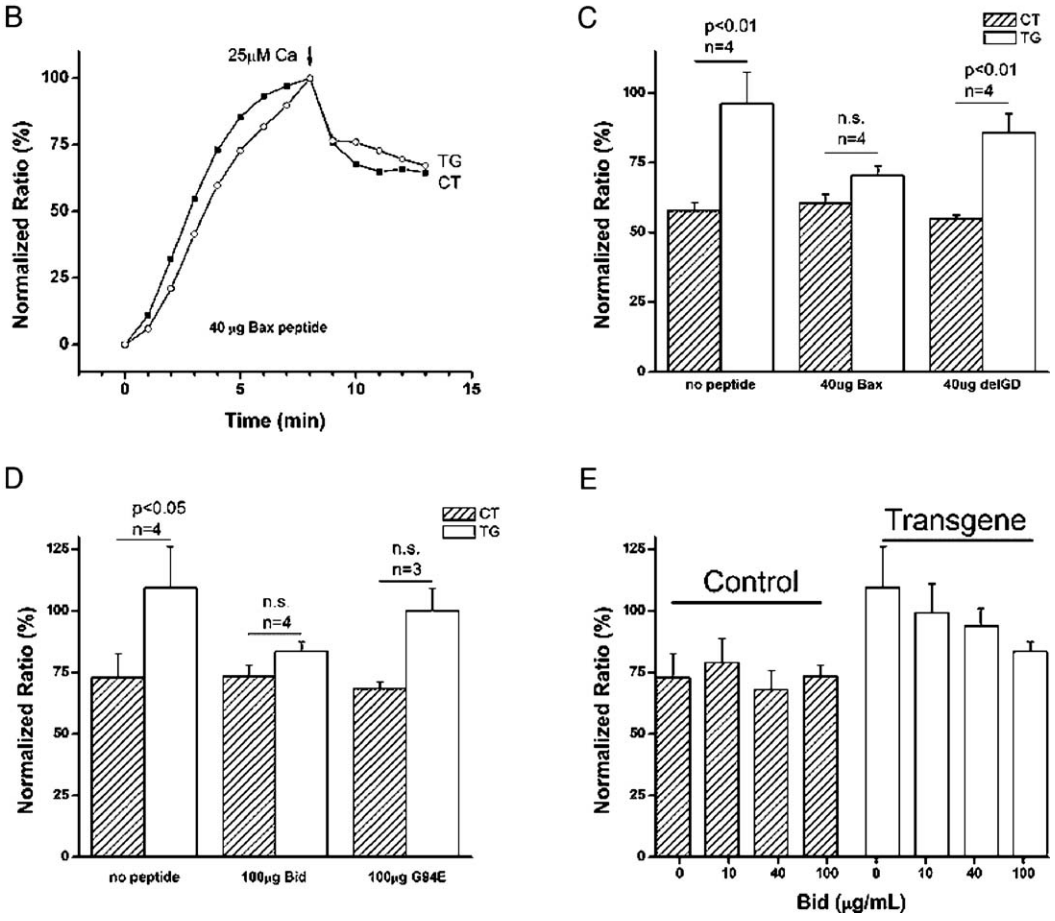



Fig. 6. Effect of Bcl-2 ligands on pore function. (Panel A) Peptides derived from the BH3 domains of Bcl-2 family members Bid and Bax were synthesized. These bind to Bcl-2 with interactions at the hydrophobic face. Note the conservation of hydrophobic groups corresponding to I86, L90, and V93 of Bid. Glycine is conserved adjacent to V93 (I66 for Bax), and changing this glycine to a bulky charged residue (G94E Bid or ΔGD Bax) disrupts the amphipathic nature of the helix and prevents binding of the peptide to Bcl-2. (Panel B) Mitochondria were incubated with the Bax BH3 peptide (40 μg/mL) starting at time zero, then challenged with calcium. Note that calcium-induced pore opening in control mitochondria is similar to experiments without BH3 peptide (see panels C, D, and E). However, the BH3 peptide normalized pore function in transgenic mitochondria, such that 25 μM calcium induced pore opening similar to control mitochondria. The JC-1 ratio at calcium addition was 4.22 for control and 6.78 for transgenic. Panel C. The extent of pore opening 5 min after calcium induction was compared across multiple experiments in the absence of peptide, with Bax BH3 peptide, or with the ΔGD Bax control peptide. The Bax BH3 peptide (40 μg/mL) restores pore function in transgenic mitochondria, while the mutant ΔGD Bax has no effect. The average JC-1 ratio at calcium addition was 4.73±0.18. (Panel D) Similar to Bax, the BH3 domain of Bid also restores normal pore function to mitochondria from transgenic mice, at a slightly higher concentration (100 μg/mL) and the Bid control peptide (G94E Bid) is ineffective. The average JC-1 ratio at calcium addition for experiments in Panels D and E was 3.35±0.37. (Panel E) The effect of the Bid peptide on pore opening is concentration dependent in mitochondria from transgenic mice, while Bid BH3 has no effect on control mitochondria.

Note that there is relative protection against pore opening in both control and transgenic hearts at 2–3 weeks of age, and in controls this protection declines after 4 weeks of age. We have previously shown that similarly, Bcl-2 levels are higher in 2-week-old mice (control and transgenic pups), and decline in control animals. Thus, the early protection may result from naturally elevated Bcl-2 levels in young animals during this period of active cardiac remodeling, but this hypothesis was not specifically tested here. The protection seen in mice with mitochondrial DNA mutations at 4 weeks of age and beyond was investigated further. Given the unexpected finding of resisted pore opening, we sought the molecular mechanism.

### 3.3. Calcium uptake

Pore opening is stimulated by calcium acting on the matrix side of the mitochondrial inner membrane [24]. Thus, the first step in calcium-stimulated pore opening is calcium uptake through the calcium uniporter [8]. A defect in calcium uptake would result in a decreased intramitochondrial calcium level, and a decreased signal for pore opening. We used the calcium-sensitive fluorophore Calcium Green 5N to measure calcium uptake in isolated mitochondria. Calcium Green 5N is excluded from mitochondria so uptake is measured as the loss of fluorescence. Fig. 4 shows representative traces where mitochondria were added followed by pulses of calcium. The initial rate of calcium uptake was the same for mitochondria isolated from controls (339±84 pmol/s/mg) or from mice with mitochondrial DNA mutations (346±190 pmol/s/mg). The amount of calcium taken up in the first minute was also the same between control (74.4±2.5%; S.E.M.) and transgenic (70.8±5.0%; S.E.M.) preparations. Therefore, resistance was not the result of decreased calcium uptake, so next we assessed whether it was due to mitochondrial Bcl-2.

### 3.4. Mitochondrial Bcl-2

We have previously shown that whole-heart Bcl-2 levels are increased in transgenic mice, [5] and here we demonstrate that Bcl-2 levels are specifically increased on the mitochondrial membrane (Fig. 5A). Recall that Bcl-2 can inhibit pore opening [14] and transgenic mitochondria are resistant to pore opening (Fig. 2). Equal loading was confirmed by probing the membrane with antibody against the voltage dependent anion channel (VDAC) (Fig. 5B).

### 3.5. Bcl-2 inhibitory peptides

The BH3 domain of Bcl-2 family proteins (e.g. Bax and Bid) forms an amphipathic helix, which can bind to a hydrophobic groove in Bcl-2 [25] and reverse protection against pore opening [26]. We designed peptides corresponding to the BH3 domain of Bax and Bid, as well as two control peptides [16,27] (Fig. 6A) and used these to determine if the inhibition of pore opening in mitochondria from transgenic mice could be normalized with Bcl-2 antagonistic peptides. The Bax BH3 peptide prevents the protection from pore opening in mitochondria from mice with mitochondrial DNA mutations (Fig. 6B and C). Thus, the addition of a Bcl-2 ligand known to disrupt pore regulation by Bcl-2 family members [26] normalizes pore function. Pore normalization is also achieved using a different BH3 peptide (derived from Bid; Fig. 6D). The specificity of pore restoration was confirmed using control peptides (see Fig. 6A for sequences). These control peptides do not inhibit Bcl-2-family members [16] and likewise did not normalize pore function (Fig. 6C and D). The reversal of protection by the BH3 peptides was concentration dependent for both Bid (Fig. 6E) and Bax (not shown) BH3 peptides. Thus pore inhibition is reversed by Bcl-2 ligands and the data are consistent with pore inhibition mediated by the increased Bcl-2 protein present on transgenic mitochondria.

## 4. Discussion

Apoptotic cell death has been shown to contribute to myocyte loss in aging, [28] as well as dilated cardiomyopathy, congestive heart failure, [29] and myocardial infarction [30]. Increased cell death can increase the severity of heart disease while inhibition of the mitochondrial permeability transition pore with cyclosporin A decreases apoptosis and heart dysfunction from ischemia–reperfusion [31]. Similarly, mice lacking cyclophilin D, a component of the pore and target for cyclosporin A, suffer a smaller infarct size after cardiac ischemia–reperfusion injury [32,33]. Interestingly, the protection from cell death was to necrotic death, triggered by calcium and/or oxidative stress [32,33].

Sublethal cardiac insults lead to protection from future insults, called physiologic preconditioning. Preconditioning can prevent apoptosis and decrease the severity of myocardial infarction [34]. Further, preconditioning inhibits the mitochondrial permeability transition pore [35] and one component of preconditioning may be mediated by Bcl-2 [36]. During aging, the loss of cardiac myocytes [28] appears to be countered by a protective response that may spare myocytes and preserve function. This altered balance of pro- and antiapoptotic signals is kept, in part, by the Bcl-2 family proteins [37]. Thus, regulation of the mitochondrial permeability transition pore and cell death is important in disease and adaptation of the heart, and may modulate cardiac disease.

Here, we have studied mice with accelerated mutagenesis of the mitochondrial DNA in the heart. Changes present in this model illustrate the pathogenic potential of mitochondrial DNA mutations at levels commonly found in aging. There remain differences, though, between mice undergoing natural aging and mice with accelerated mitochondrial mutagenesis. For instance, with aging comes increased oxidative damage, while we have previously reported there is no increase in oxidative damage or stress in these mice [5]. Further, these mice exhibit a marked upregulation of Bcl-2 protein, while studies of aging rats have found either significantly increased [37,38], unchanged [39], or an apparent trend toward decreased Bcl-2 protein levels [40,41] in 24-month-old rat hearts versus 6-month-olds. It is not clear why similar methods yielded different results, except perhaps the effort by Centurione et al. [38], which used Wistar rats as opposed to Fischer 344 rats in all others. It is possible that the animals used had differing degrees of coronary artery disease. This uncertainty illustrates the need for models of aging that can separate cause and effect. The transgenic mice used in the present study have

increased mitochondrial DNA mutations in the absence of age-related oxidative stress or other confounding variables. This allows for the study of the effects of mitochondrial DNA mutations per se. For the same reason then, the reader should be cautioned that this is not a model for all of aging, only for the effects of mitochondrial DNA mutations. Further, these mutations are accumulated over a period of weeks, as opposed to years in natural aging.

In mice with mitochondrial DNA mutations, we also see an altered apoptotic balance. Specifically, we have demonstrated increased cytochrome *c* release and myocyte death, as well as an increase in antiapoptotic Bcl-2 [5]. In the present study, we evaluated the function of the mitochondrial permeability transition pore in these mice. The expectation was that increased pore opening would explain the increase in cytochrome *c* release and cell death. Instead, we found an inhibition of pore opening. Resistance was most evident at low calcium (500 nmol/mg mitochondrial protein) and was absent at higher calcium loads (2000 nmol/mg).

There is correlation of pore inhibition with Bcl-2 in the literature [14–17,42]. Further, experiments on isolated channels in the mitochondrial membrane with characteristics similar to the pore (mitochondrial megachannel) show inhibition of calcium-induced opening [43]. Here, we showed a marked increase in Bcl-2 immunoreactivity on the mitochondria. Bcl-2 is known to act at the mitochondrial membrane to inhibit pore opening [42] though not in all cases [44]. Of interest, previous data demonstrated elevated Bcl-2 levels in very young control and transgenic pups (2 weeks old), with the Bcl-2 level of control mice declining to low levels by 4 weeks of age and beyond. In contrast, mice with elevated mitochondrial DNA mutations had consistently elevated levels of Bcl-2 [5]. The sensitivity of isolated mitochondria to calcium-induced pore opening was inhibited in both control and transgenic mice at 2–3 weeks of age, and this inhibition persisted in transgenic mice while control mice became more sensitive (Fig. 3). This is further correlative evidence of a role for Bcl-2 in pore inhibition.

To determine if Bcl-2 was inhibiting pore function, we treated mitochondria isolated from mice 4 weeks of age or older with Bcl-2 antagonistic ligands and showed restoration of pore function. We take this to show that Bcl-2 is functional at the level of the mitochondrial membrane, and that Bcl-2 acts to inhibit opening of the mitochondrial permeability transition pore in mice with mitochondrial DNA mutations. An alternate interpretation is that a different Bcl-2 family member that also binds the antagonistic ligands is mediating the resistance. We have separately shown that Bcl-$_{XL}$ and Bfl-1 proteins are also upregulated in transgenic mice [45]. However, the level of Bcl-$_{XL}$ protein at the mitochondria is not different, suggesting that the role of Bcl-$_{XL}$ in this model is non-mitochondrial. The role of Bfl-1, including its localization, has yet to be studied in this model, and it remains possible that Bfl-1 contributes to the findings reported here. Interestingly, it has been shown that the BH-3 peptides exhibit discriminatory binding preferences [46]. In our system, the concentration dependence of the Bid peptide suggests that functional binding occurs in the range of 4.4 to 44 μM (10–100 μg/mL), consistent with the IC50 of Bid for Bcl-2 of 6.8 μM found by Huang and colleagues. Still, we would caution not to overinterpret this finding, as our functional system is very different than their binding system, and further, the length of BH3 peptide used was not the same. The timing, localization, and degree of Bcl-2 upregulation, as well as the peptide inhibitor data are consistent with the model that Bcl-2 acts at the mitochondrial membrane to inhibit pore opening in mice with mitochondrial DNA mutations.

There remains controversy regarding the mechanism of release of mitochondrial cytochrome *c*. Mice with cardiac mitochondrial DNA mutations have increased release of cytochrome *c* and increased apoptosis in the heart [12]. While pore opening can cause cytochrome *c* release [7,32,33], release can also occur independent of pore opening [47,48]. Thus, the finding here that pore opening is inhibited is not necessarily at odds with the increased cytochrome *c* release and increased cell death in these mice.

In summary, Bcl-2 is upregulated in the hearts of mice with cardiac mitochondrial DNA mutations, and localizes to mitochondrial membranes. The current study provides supporting evidence that Bcl-2 is active at the mitochondria and inhibits opening of the permeability transition pore. Regulation of pore opening may be important in pathogenesis in these mice, given that cyclosporin A treatment protects mice against disease [49].

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